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Supporting Online Material for

Ubiquitinated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis

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Supporting Online Material

Materials and Methods:

Brain tissue collection and neuropathological assessment.

Frozen brain tissues and fixed, paraffin-embedded tissue blocks were obtained from following institutions: the Center for Neurodegenerative Disease Research (CNDR) Brain Bank at the University of Pennsylvania, USA; Center for Neuropathology and Prion Research Brain Bank at the University of Munich, Germany; Department of Pathology, University of British Columbia, Canada (source of UBC-17); Department of Neurosciences, University of California San Diego, USA (source of HDDD2). Consent for autopsy was obtained from legal representative from all subjects in accordance with local Institutional Review Boards. Neuropathological diagnostic assessment of FTLD-U, ALS, AD, Parkinson's disease (PD), multiple system atrophy (MSA), progressive supranuclear palsy (PSP), neuronal intermediate filament inclusion disease (NIFID) and neuropathologically normal controls (CO) was performed in accordance with published guidelines (*1-6*).

Antibodies.

Antibodies used in this study included: 1) anti-ubiquitin antibodies: mouse MAb 1510 (Chemicon, Temecula, CA), rabbit polyclonal antibody (Dako, Carpinteria, CA), mouse MAb Ub1B4 (unpublished, CNDR), 2) anti-tau antibodies: mouse MAbs T14 and T46 (CNDR) (*7, 8*), mouse MAb PHF-1 (*9*) (a gift from Dr. P. Davies), 3) anti-TDP-43

antibodies: rabbit polyclonal antibody (ProteinTech Group, Chicago, IL); mouse MAb 2E2-D3 (Abnova, Taipei, Taiwan), 4) anti-FTLD-U antibodies: MAbs 182 and 406 (see below for antibody production), 5) anti-α-synuclein: rat MAb 15G7 (*10*), and 6) anti-α-internexin (Zymed Laboratories Inc., San Francisco, CA)

Immunohistochemical staining.

The harvesting, fixation, and further processing of the tissue specimens used in this study were conducted as described previously (*11*). Briefly, tissue blocks from representative brain regions (frontal and temporal cortices, hippocampus, basal ganglia, medulla and spinal cord) were fixed with either 70% ethanol in 150 mM NaCl or phosphate-buffered 3.65% formaldehyde, and paraffin-embedded. Immunohistochemistry was carried out as described (*11*) with sections pretreated with formic acid (5 min) to enhance anti-TDP-43 immunoreactivity. Frozen sections (10 µm) from FTLD-U brains were used for screening of newly generated MAbs. Briefly, frozen sections were air-dried (30 min), fixed in ice-cold acetone (5 min) and air-dried (30 min) again. Endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol (15 min) and immunohistochemistry performed as described for paraffin-embedded sections. Double-labeling immunofluorescence was performed as previously described (*11*) using Alexa Fluor 488 and 594 conjugated secondary antibodies (Molecular Probes, Eugene, OR).

Sequential biochemical fractionation, dephosphorylation and immunoblot analysis:

Post-mortem brain tissue was dissected, weighed, and sequentially extracted with buffers of increasing strength as previously described (*11*). Briefly, gray matter was extracted at 5 mL/g (volume/weight) with low salt (LS) buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 10% sucrose, and a cocktail of protease inhibitors), high salt-Triton (TX)

buffer (LS + 1% Triton X-100 + 0.5M NaCl), myelin floatation buffer (TX buffer containing 30% sucrose), and sarkosyl (SARK) buffer (LS + 1% N-Lauroyl-sarcosine + 0.5 M NaCl). The SARK insoluble materials were extracted in 0.25 mL/g urea buffer (7M urea, 2M thiourea, 4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris, pH 8.5). Proteins were resolved in Tris-glycine 5-20% gradient SDS-PAGE, transferred to nitrocellulose and probed with primary and secondary antibodies (horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Jackson ImmunoReasearch, West Grove, PA)). Blots were developed with Renaissance Enhanced Luminol Reagents (NEN Life Science Product, Inc., Boston, MA), and digital images were acquired using a Fujifilm Intelligent Darkbox II (Fuji Systems USA, Stamford, CT). Where indicated, TDP-43 was dephosphorylated by dialysis (50 mM Tris, 0.2 mM EDTA, pH 8.0) and treated with *Escherichia coli* alkaline phosphatase (Sigma, St. Louis MO) for 2h at 56˚C.

Generation of novel MAbs.

Murine MAbs 406 (case #18) and 182 (case #11) were generated using high M_r (>250 kD) and M_r 20-30 materials, respectively, from urea fractions of FTLD-U frontal cortex as immunogen as previously described (*11*). Briefly, urea fractions (100-150 μg protein/mouse) were separated using 5-20% gradient SDS-PAGE, and the portion of the gel containing proteins with M_r > 250 kD (including the stacking gel) or M_r 20-30 was minced, homogenized in phosphate-buffered saline, emulsified with incomplete Freund's adjuvant, and injected subcutaneously into BALB/c mice. Boost injections (25-50 μg protein/mouse) were made on days 21, 35, and 49, followed by intraperitoneal injection of immunogens without adjuvant on day 63. Fusion was conducted on day 66 using Sp2

myeloma cells as fusion partner. Resulting hybridoma supernatants were screened by immunohistochemistry on paraffin-embedded and frozen sections of FTLD-U cortex known to contain UBIs. All positive MAbs were determined to be of the IgM class using standard light and heavy chain antibody subtype analysis.

Two-Dimensional (2D)-PAGE

2D-PAGE was performed with the ZOOM® IPGRunner™ system (Invitrogen Corp., Carlsbad, CA) using pH 3-10L or pH 3-10NL strip for the first dimension separation and 4-12% Bis-Tris PAGE for the second dimension according to manufacturer's protocol. Gels were either stained with Colloidal Blue (Invitrogen Corp., Carlsbad, CA) or transferred to nitrocellulose membrane and immunblotted with MAbs 406 or 182. Protein spots corresponding to immuno-positive spots were excised from gels, digested with sequencing grade trypsin and the peptides separated by nano liquid chromatography on a C_{18} capillary column. Eluted peptides were sequenced on line with a nanospray Qstar-XL mass spectrometer (Applied Biosystems, Foster City, CA). Data were acquired and analyzed with Analyst QS software, and Mascot dll script was used for database search. Protein total score >70 with confidence >95% was accepted as positive identification.

Immunoprecipitation

Urea fractions were dialyzed into RIPA buffer (0.1% SDS, 1% NP40, 0.5% sodium dexoycholate, 5 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 8.0), pre-absorbed with Protein A Sepharose, and immunoprecipitated with polyclonal TDP-43 antibody conjugated to Protein A Sepharose CL-4B (GE Healthcare Bio-Sciences, Piscataway, NJ). Immunoprecipitated proteins were eluted with SDS sample buffer (10 mM Tris, pH

6.8, 1 mM EDTA, 40 mM DTT, 1% SDS, 10% sucrose), resolved by 5-20% SDS-PAGE and analyzed by immunoblot as described above.

Table S1: Demographic characteristics of FTLD-U cases used in this study

Case No	Diagnosis	Age at death	Sex	Duration	Dementia	MND	Family history
1	**FTLD-U 1**	62	F	5	yes	no	no
2	**FTLD-U 1**	71	M	8	yes	no	no
3	**FTLD-U 1**	92	M	3	yes	no	no
4	**FTLD-U 1**	77	M	12	yes	no	no
5	**FTLD-U 1**	69	F	6	yes	no	yes
6	**FTLD-U 1**	77	M	nr	yes	no	no
7	**FTLD-U 1**	76	F	11	yes	no	no
8	**FTLD-U 1**	68	F	7	yes	no	no
9	**FTLD-U 1**	64	M	10	yes	no	no
10	**FTLD-U 1**	81	F	2	yes	no	no
11	**FTLD-U 1**	54	M	7	yes	no	no
12	**FTLD-U 1**	73	M	10	yes	no	no
13	**FTLD-U 2**	57	M	3	yes	yes	yes
14	**FTLD-U 2**	54	M	2	yes	yes	no
15	**FTLD-U 2**	54	F	7	yes	no	yes
16	**FTLD-U 2**	61	F	4	yes	no	yes
17	**FTLD-U 2**	67	M	10	yes	yes	yes
18	**FTLD-U 2**	41	M	6	yes	no	yes
19	**FTLD-U 2**	44	M	nr	yes	yes	no
20	**FTLD-U 2**	57	F	7	yes	yes	yes
21	**FTLD-U 2**	48	M	9	yes	yes	no
22	**FTLD-U 2**	42	F	3	yes	yes	no
23	**FTLD-U 2**	67	M	2	yes	yes	no
24	**FTLD-U 2**	47	F	2	yes	no	no
25	**FTLD-U 2**	59	M	1	yes	no	no
26	**FTLD-U 2**	72	M	nr	yes	no	no
27	**FTLD-U 3**	nr	F	nr	yes	no	no
28	**FTLD-U 3**	75	F	3	yes	no	no
29	**FTLD-U 3**	62	F	5	yes	no	yes
30	**FTLD-U 3**	65	M	6	yes	yes	yes
31	**FTLD-U 3**	79	F	5	yes	yes	yes
32	**FTLD-U 3**	76	F	7	yes	no	yes
33	**FTLD-U 3**	77	F	11	yes	no	yes
34	**FTLD-U 3**	69	F	7	yes	no	yes
35	**FTLD-U 3**	55	M	2	yes	no	no
36	**FTLD-U 3**	73	F	6	yes	no	yes
37	**FTLD-U 3**	76	M	7	yes	no	no
38	**FTLD-U 3**	63	F	11	yes	yes	no
39	**FTLD-U 3**	49	F	3	yes	no	yes
40	**FTLD-U 3**	59	M	10	yes	yes	no
41	**FTLD-U 3**	48	M	2	yes	yes	no
42	**FTLD-U 3**	53	F	2	yes	yes	no
43	**FTLD-U 3**	53	M	3	yes	yes	no
44	**FTLD-U 3**	72	F	3	yes	no	no
45	**FTLD-U 3**	60	F	2	yes	no	no
46	**FTLD-U 3**	37	M	2	yes	yes	no
47	**FTLD-U 3**	65	M	1	yes	yes	no
48	**UBC-17**	60	F	6	yes	yes	yes
49	**UBC-17**	61	M	4	yes	no	yes

50	**HDDD2**	57	F	5	yes	no	yes
51	**HDDD2**	65	M	6	yes	no	yes
52	**HDDD2**	64	M	8	yes	no	yes
53	**HDDD2**	74	F	6	yes	no	yes
54	**ALS**	56	F	nr	no	yes	yes
55	**ALS**	56	M	2	no	yes	no
56	**ALS**	52	M	nr	no	yes	no
57	**ALS**	83	M	3	yes*	yes	no
58	**ALS**	55	F	nr	no	yes	no
59	**ALS**	57	M	2	no	yes	no
60	**ALS**	61	M	2	no	yes	no
61	**ALS**	64	F	1	no	yes	no
62	**ALS**	48	F	6	no	yes	no
63	**ALS**	68	F	nr	no	yes	no
64	**ALS**	80	F	nr	no	yes	no
65	**ALS**	73	F	6	no	yes	no
66	**ALS**	61	M	2	no	yes	no
67	**ALS**	55	M	3	no	yes	no
68	**ALS**	81	F	2	no	yes	no
69	**ALS**	60	M	5	no	yes	no
70	**ALS**	77	F	1	no	yes	no
71	**ALS**	68	M	3	no	yes	no
72	**ALS**	51	M	2	no	yes	no

UBC-17 and HDDD2 are families with published linkage to chromosome 17 (*12*, *13*).

* This patient also had severe AD pathology (CERAD C, Braak & Braak stage V-VI). Age and disease duration are given in years. Abbreviations: M = male, F = female, nr = not recorded.

Figure Legends:

Figure S1: Identification of protein spots for LC-MS/MS analyses.

Protein samples from urea fraction of case #18 (**A, B**) and case #11 (**C, D**) were analyzed by 2D-PAGE. Duplicate gels were used for immunoblot analyses with MAbs 406 (**A**) and 182 (**C**), and for protein staining with colloidal coomassie blue (**B** and **D**). Arrows point to protein spots excised for trypsin-digestion and LC-MS/MS analyses.

Figure S2: Co-Localization of anti-TDP-43 with Mab 182, Mab 406 and anti-ubiquitin in FTLD-U

Double-label immunfluorescence demonstrating immunolabeling of long neuritic profiles in FTLD-U Type 1 with Mab 182 (**A**) and anti-TDP-43 (**B**), cytoplasmic inclusions in FTLD-U Type 2 with Mab 406 (**D**) and anti-TDP-43 (**E**), UBIs in FTLD-U Type 3 with anti-ubiquitin (**G**) and anti-TDP-43 (**H**), UBIs in HDDD2 with anti-ubiquitin (**J**) and anti-TDP-43 (**K**). Overlays demonstrating co-localization of the corresponding immunostainings are shown in (**C**, **F**, **I**, **L**). All sections are from frontal cortex. Scale bar in (**A**) corresponds to 50 µm (**A-L**).

Figure S3: TDP-43 immunoreactivity is detected in UBIs of all FTLD-U cases but not in inclusions of other neurodegenerative diseases. Immunohistochemistry with anti-TDP-43 antibody of temporal cortex of FTLD-U Type 1 (**A, E**), Type 2 (**B, F**), Type 3 (**C, G**), and FTDP-17U (UBC-17 family, **D, H**) demonstrates robust labeling of UBIs. No TDP-43 immunoreactivity was observed in tau-positive inclusions (detected by PHF1) in AD (**I, J**), PSP (**K, L**), and FTDP-17T (**M, N**), α-synuclein-positive Lewy

bodies in PD (**O, P**) and glial cytoplasmic inclusions in MSA (**Q, R**) or α-internexin-positive neuronal inclusions in NIFID (**S, T**). Scale bar in **A** corresponds to 50 μm (**A-D, I-N, Q-T**), 25 μm (**E-H**) and 20 μm (**O, P**).

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Figure S1

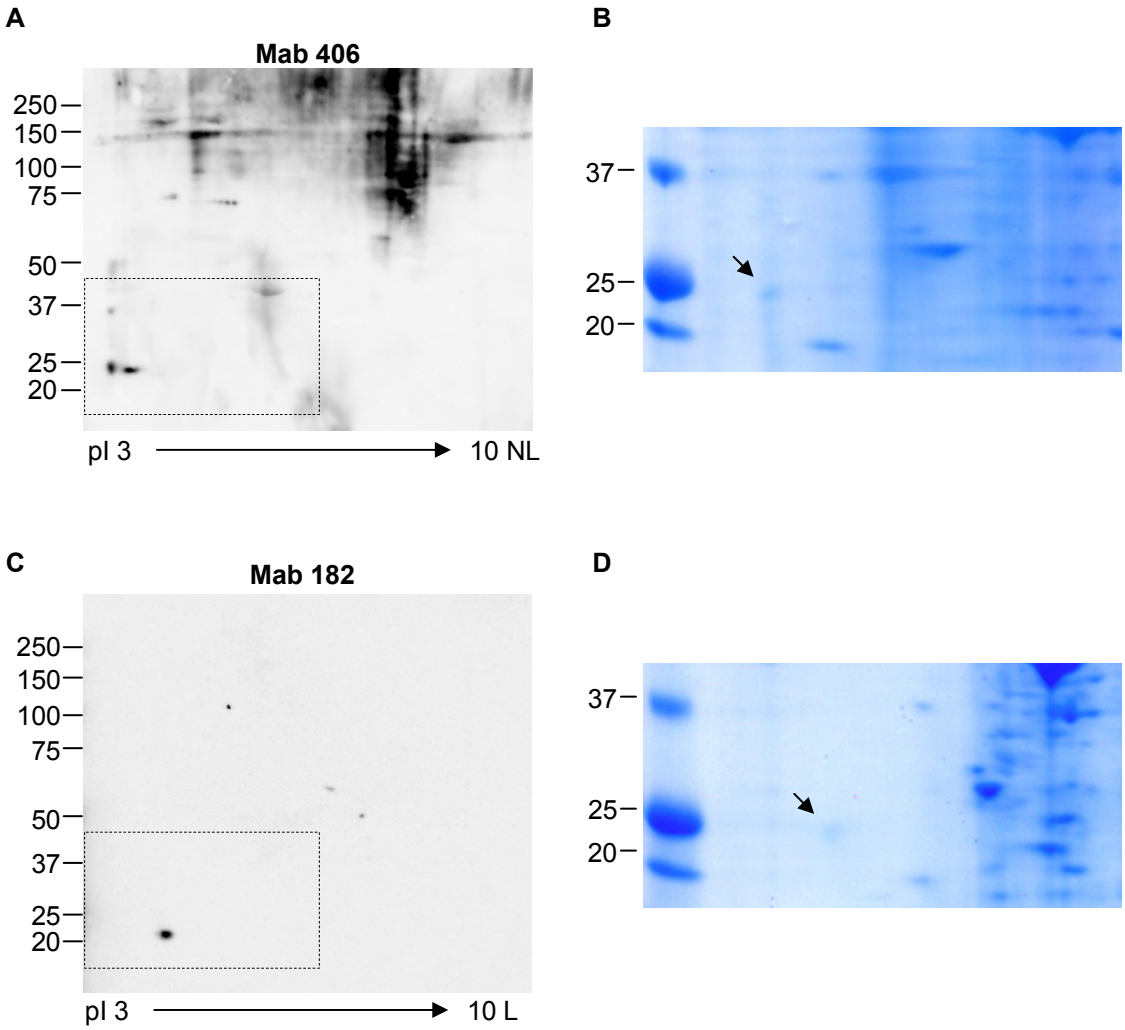

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Figure S2

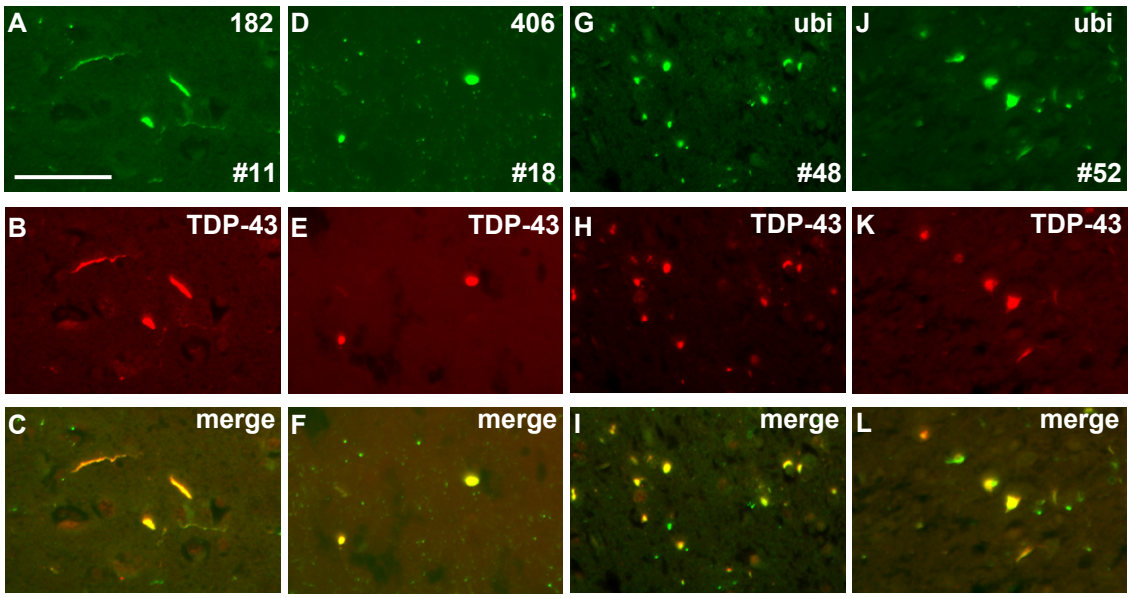

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Figure S3